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METHODS FOR ESTIMATION OF VITAMIN C IN FOODSTUFFS

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METHODS FOR ESTIMATION OF VITAMIN C IN FOODSTUFFS

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Indian Standard

METHODS FOR ESTIMATION OF VITAMIN C IN FOODSTUFFS

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 30 November 1970, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Vitamins are required to be assessed in a large number of foodstuffs, such as dairy products, animal feeds, processed cereals and other foodstuffs. Moreover, different methods of vitamin assays are used in different laboratories. Therefore, with a view to establishing uniform procedures and also for facilitating a comparative study of results, ISI is bringing out a series of standards on vitamin assays. These would include chemical and microbiological methods, wherever applicable.

0.3 This standard covers two methods, namely, 2,6-dichlorophenol indophenol method and 2,4-dinitrophenylhydrazine method, commonly used for estimation of vitamin C in foodstuffs. The 2,4-dinitrophenylhydrazine method is particularly suitable when the sample contains a very low amount of ascorbic acid. The method is not very specific in case of processed foods that contain high concentrations of carbohydrates and have been heated.

0.4 In the preparation of this standard, considerable assistance has been derived from a number of books and publications. However, the methods included in this standard are predominantly those which have been tried in various laboratories in the country. Thus the methods prescribed in this standard are mainly based on practical experience within the country.

0.5 In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS:2-1960*.

1. SCOPE

1.1 This standard specifies methods for estimation of Vitamin C (ascorbic acid) in foodstuffs.

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*Rules for rounding off numercial values (*revised*).

2. QUALITY OF REAGENTS

2.1 Unless specified otherwise, pure chemicals shall be employed in tests and distilled water (*see* IS : 1070-1960*) shall be used when the use of water as a reagent is intended.

Note — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the results of analysis.

3. PREPARATION OF THE ASSAY SAMPLE

3.0 The technique used for preparing the material for the analysis is mostly common to every vitamin determination. It should be ensured that the sample taken for the assay is representative of the whole and any deterioration of the vitamin to be examined is prevented.

3.1 Powders and liquids should be mixed thoroughly until homogeneity is achieved. Dry materials, such as bread, biscuits and grains, should be ground and mixed thoroughly.

3.2 Wet or fresh material may be minced with a knife or scissors, or homogeneized in a blender, if necessary, in the presence of the extracting solvent.

4. 2,6-DICHLOROPHENOL INDOPHENOL METHOD

4.0 Principle — The hydrogen atoms of the two enolic groups of ascorbic acid (Vitamin C) can be oxidized readily, as this compound is a strong reducing substance. Measurement of this reducing property of ascorbic acid under appropriate conditions is the basis of several methods for determining the quantity of ascorbic acid.

4.0.1 Of the various oxidation-reduction methods, those based upon the reduction of indophenol have been found to be the most satisfactory. The compound 2, 6-dichlorophenol indophenol is blue in alkaline conditions and pink in acidic conditions. It is reduced by ascorbic acid to a leukoform. A dilute solution of the dye is added dropwise from a burette to an acid extract of vitamin source. A pink colour that persists for 15 seconds after the addition of one drop of dye is the end point of the titration. Interfering reducing substances are phenols, sulphydryl compounds, thiosulphate, sulphites, ferrous, cuprous and stannous ions. Interference from phenols and sulphydryl compounds is diminished by carrying out the reduction below *p*H value of 4, since most of the phenolic compounds do *not* reduce indophenol at low *p*H and reduction by the sulphydryl group is so low that a correction can be obtained. Thiosulphates, ferrous and cuprous compounds reduce indophenol and their presence in appreciable quantities lead to falsely high values of ascorbic acid. Another group of interfering substances in oxidation reduction methods are the compounds loosely classified together and called 'reductones'.

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*Specification for water, distilled quality (*revised*).

NOTE 1 — Interference from sulphides, sulphites and thiol compounds can be overcome by treatment with formaldehyde. In presence of these substances, the extract is rendered acidic to pH 0·6 and formaldehyde is added. This would react with all sulphides, sulphites and thiol compounds leaving the ascorbic acid free. Then the titration in the usual method is carried out.

NOTE 2 — In case of turbid or highly coloured solutions, the estimation can be carried out with the help of a photo-electric colorimeter.

4.1 Reagents

4.1.1 *Trichloroacetic Acid (TCA) Reagent* — 10 percent. Dissolve 10 g of TCA in 100 ml of water.

4.1.2 *Metaphosphoric Acid* — 5 percent.

4.1.3 *Standard Ascorbic Acid Solution* — Weigh approximately 100 mg of USP ascorbic acid reference standard or equivalent IP Standard accurately to ± 0·1 mg. Transfer it to a 100-ml glass-stoppered graduated flask, dissolve and dilute to the mark with the TCA (*see* **4.1.1**) or metaphosphoric acid (*see* **4.1.2**).

4.1.4 *Standard Indophenol Solution* — Dissolve 50 mg of sodium 2,6-dichlorobenzenone indophenol, that has been stored in a desiccator over soda lime, in 50 ml of water to which 42 mg of sodium bicarbonate has been added. Shake vigorously and, when the indophenol has completely dissolved, dilute it to 200 ml with water. Filter the solution through a fluted filter paper into an amber glass-stoppered bottle. Keep the bottle stoppered, out of direct sunlight, and store in a refrigerator. Decomposition products that make the end point indistinct occur in some batches of dry indophenol and also develop with time in stock solution. Add 5 ml of the TCA reagent or metaphosphoric acid containing excess of ascorbic acid to 15 ml of the indophenol solution. If the reduced solution is not practically colourless, discard and prepare a new stock solution. If the dry indophenol dye is proved to be of a bad quality, obtain a new sample.

4.1.4.1 *Standardization of indophenol solution* — Standardize the indophenol solution immediately after it has been prepared as follows:

> Transfer three 2-ml aliquots of the standard ascorbic acid solution to each of the three 50-ml Erlenmeyer flasks containing 5 ml of the TCA reagent or metaphosphoric acid. Titrate rapidly with the indophenol solution from a 25-ml burette until light but distinct rose-pink colour persists for at least five seconds. (Each titration should require approximately 15 ml of indophenol solution and the titrations should check within 0·05 ml.) In the same way, titrate three blanks composed of 7 ml of the TCA reagent or metaphosphoric acid along with water, the volume of which is approximately equivalent to the volume of the indophenol solution used in the direct titration.

Calculate and express the concentration of the indophenol solution as milligrams of ascorbic acid equivalent to one millilitre of the indophenol solution. Standardize the indophenol solution daily with freshly prepared standard ascorbic acid solution.

4.2 Procedure

4.2.1 *Preparation of Sample* — Grind an accurately weighed sample (about 5 g) in a mortar with acid washed sand using TCA reagent or metaphosphoric acid and transfer into 100-ml graduated flask. Shake the mixture thoroughly and make up the volume to 100 ml with TCA reagent or metaphosphoric acid. Filter immediately through a fluted filter paper. The final concentration of ascorbic acid in the extract should be 10 to 15 µg per ml.

4.2.2 *Determination of Vitamin C (Ascorbic Acid)* — Take 10 ml of the filtrate (*see* **4.2.1**), and titrate rapidly with the indophenol solution. Carry out a blank determination with 11 ml of the reagent along with water sufficient to make the volume of the mixture equivalent to 15 ml plus the volume of the indophenol solution required in the direct titration (*see* **4.1.4.1**).

4.3 Calculation

4.3.1 Calculate the vitamin C content in the sample as follows:

$$\text{Vitamin C content, mg, per 100 g of the sample} = \frac{A \times B \times 1\,000}{W}$$

where

A = volume in ml of the indophenol solution used for titration,

B = weight in mg of the ascorbic acid equivalent to one millilitre of the indophenol solution, and

W = weight in g of the sample taken for the test.

5. 2,4-DINITROPHENYLHYDRAZINE METHOD

5.0 Principle — The ascorbic acid is converted to dehydroascorbic acid by oxidizing agents, such as, activated charcoal, etc, and on treatment with 2,4-dinitrophenylhydrazine, a 2,4-dinitrophenylhydrazone is formed. This gives a reddish colour with 85 percent sulphuric acid and the intensity of the colour follows Beer's law. This method measures not only ascorbic acid but also dehydroascorbic acid (biologically active) and dikotogulonic acid (biologically inactive).

NOTE — Reductones, which are formed by boiling sugars in a mildly alkaline solution or by maintaining foods at 60 to 70°C, would also react with 2,4-dinitrophenylhydrazine. In case they are present, the formed hydrozones should be purified by chromatography on acid alumina.

5.1 Apparatus

5.1.1 *Photoelectric Colorimeter*

5.2 Reagents

5.2.1 *Standard Ascorbic Acid Solution* — Weigh accurately about 50·0 mg of reference standard ascorbic acid and dissolve in the acid solution used for extraction. Make the volume to 50 ml. One millilitre of this solution is diluted to 100 ml. This working standard solution will contain 10 µg/ml.

5.2.2 *Sulphuric Acid Solution* — 9 N. Add cautiously 250 ml of concentrated sulphuric acid (sp gr 1·84) to 700·0 ml of distilled water kept in ice. When cool, dilute to 1 litre.

5.2.3 *2,4-dinitrophenylhydrazine (DNPH)* — Dissolve 2·0 g of 2, 4-dinitrophenylhydrazine in 100 ml of 9 N sulphuric acid and filter through filter paper. Keep under refrigeration when not in use.

5.2.4 *Thiourea* — 10 percent. Dissolve 10 g of thiourea in 50 percent alcohol and make the volume to 100 ml with 50 percent alcohol.

5.2.5 *Trichloroacetic Acid* — 6 percent and 4 percent.

5.2.6 *Acid Washed Animal Charcoal* — Place 200 g of animal charcoal in a large flask and add 1 litre of 10 percent hydrochloric acid. Heat to boiling, then filter with suction. Remove the cake of animal charcoal to a large beaker and add one litre of distilled water, then stir and filter. Repeat this process until the filtrate is free from chloride ions (checked by silver nitrate test). Dry the animal charcoal overnight in an oven at 115 to 120°C.

5.2.7 *Sulphuric Acid* — 85 percent. To 100 ml of distilled water in a flask kept in cold, add 900 ml concentrated sulphuric acid (sp gr 1·84) carefully with constant swirling.

5.3 Procedure

5.3.1 *Extraction of the Vitamin from the Material* — Grind a known quantity of the material in a mortar with acid washed sand using 6 percent trichloroacetic acid. Adjust the amount of the extracting fluid so as to give a concentration of 5 to 10 µg ascorbic acid per millilitre of the extract. If the amount of protein present in the material is small, use 4 percent TCA instead of 6 percent. The material may also be homogenized with 6 percent TCA using a homogenizing tube and rod. It is desirable to surround the homogenizer with ice.

5.3.2 *Activated Animal Charcoal Treatment* — To 25·0 ml of the extract add approximately 0·5 g of activated animal charcoal. Shake and allow to stand for 10 minutes. Filter through filter paper (Whatman No. 42 or equivalent).

5.3.3 *Treatment of Charcoal Filtrate* — Place aliquots containing 5 to 50 µg of ascorbic acid into test tubes, make the volume in all the cases to 4·0 ml with the grinding medium. Add to each tube 0·05 ml of thiourea reagent and 1 ml of 2 percent DNPH. A tube containing 4·0 ml of the extracting medium serves as the reagent blank. Incubate for 3 h at 37°C. Place the tubes at the end of the incubation period in crushed ice and add 5·0 ml of 85 percent sulphuric acid drop by drop from a burette. Shake the tubes thoroughly. Allow the tubes to stand for 30 minutes at room temperature before reading the colour at 540 mµ adjusting the Instrument to zero with the blank.

5.3.4 Treat a standard solution of ascorbic acid in the range of 5 to 50 microgram concentration in the same way as the experimental and take the readings with the standard. Compare the readings with the unknown and calculate the concentration of ascorbic acid in the aliquot (*see* **5.3.3**).

NOTE — In case reductones and other interfering substances are present, the hydrozones formed after incubation are completely extracted with ethyl acetate and purified by chromatography. Columns of acid alumina are washed with ethyl acetate containing 2 percent (*v/v*) acetic acid. The ethyl acetate extract of the hydrozones is added to the alumina column. The chromatogram is developed with ethyl acetate acetic acid solvent. Unchanged 2,4-dinitrophenylhydrazine is first eluted. The osazone of dehydroascorbic acid is then eluted from column by ethyl acetate. Other hydrozones from interfering substances are firmly held on the column. The eluted solution of osazone of dehydroascorbic acid is then evaporated to dryness under vacuum. The osazones are dissolved in 1 ml ethyl acetate to which is added 5 ml sulphuric acid (50 percent *w/v*). The concentration of dehydroascorbic acid is determined from the absorption at 530 mµ, and compared with standard solution of dehydroascorbic acid (prepared from ascorbic acid) and treated in the same way.

5.4 Calculation — Calculate the quantity of Vitamin C (ascorbic acid) in the amount of sample taken (*see* **5.3.1**) and express the results as µg of Vitamin C (ascorbic acid) per gram of the food sample on dry basis or wet basis as the case may be.